Exhibit B

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Genetics

Homologous recombination can restore normal immunoglobulin production in a mutant hybridoma cell line

(marker rescue/mammalian gene targeting/immunoglobulin engineering)

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ABSTRACT **We report here the occurrence of homologous recombination between transferred and chromosomal immunoglobulin genes. Specifically, we have corrected a chromosomal immunoglobulin gene mutation by transferring pSV2neo vectors encoding the constant region of the immunoglobulin μ heavy chain to mutant hybridoma cells that bear a 2-base-pair deletion in the third constant region exon of their chromosomal μ gene. After DNA transfer, we detected G418-resistant transformants that produce normal IgM. Analysis of the DNA structure of the μ gene in these transformants indicates that in four of five cases the μ gene has been restored as a result of the integration of a single copy of the transfer vector by a reciprocal homologous recombination event; the fifth case seems to have resulted from gene conversion or double crossover. These results suggest that this technology might be adapted for mapping immunoglobulin gene mutations by marker rescue and for more convenient engineering of specifically altered immunoglobulin.**

The usual methods for identifying the regulatory elements of the immunoglobulin genes have depended on assaying expression from transferred DNA. Several such elements have been defined by comparing expression from specifically altered immunoglobulin genes (1). A possible weakness of this approach is that the transferred genes are assayed in an abnormal context, either integrated at abnormal chromosomal sites or in an unintegrated form. If a regulatory element were to depend on the normal chromosomal context or to lie outside the gene segment that is assayed, the usual gene transfer methods would be inadequate.

In microbial systems, these problems are avoided because the chromosomal genes can be modified by homologous recombination with a segment of transferred DNA. Such a technique would be very useful for introducing precise changes into the chromosomal immunoglobulin genes as well as for mapping regulatory mutations, thus providing an important tool for analyzing immunoglobulin gene expression. To assess the feasibility of this approach, we have tested for homologous recombination between transferred and chromosomal immunoglobulin genes by using, as recipient cells, a mutant hybridoma with a frameshift mutation in the DNA encoding the constant region of the μ heavy chain (C_μ region) and vectors that bear DNA encoding the normal C_μ region. Here we report that homologous recombination can occur between transferred and chromosomal μ genes so as to regenerate a wild-type μ gene sequence and restore normal IgM production.

MATERIALS AND METHODS

General Techniques. The cell lines and methods for cell culture and DNA analysis have been described (2–4).

Gene Transfer. The construction of the pRCμ and pTCμ vectors carrying the subfragment of the wild-type μ gene is described in the text. The appropriate vector DNA (50 μg) was linearized by *Bam*HI digestion, extracted with phenol, precipitated with ethanol, and resuspended in 50 μl of phosphate-buffered saline (PBS) (2). Mutant igm482 cells were harvested by centrifugation and were washed, and 2×10^7 cells were resuspended in 0.45 ml of PBS. Prior to electroporation, the plasmid DNA and igm482 cells were kept on ice. The plasmid DNA and igm482 cells were mixed, subjected to a 700-V, 25-μF pulse, and placed on ice for 10 min. The cells were then transferred to 40 ml of Dulbecco's modified Eagle's medium (DMEM) containing 13% heat-inactivated fetal calf serum and 3.5×10^{-4}% 2-mercaptoethanol (2, 3) and were placed at 37°C. Cell viability was determined by trypan blue exclusion after ≈12 hr of incubation.

IgM Analysis. Plaque-forming cells (PFC) were detected by lysis of 2,4,6-trinitrophenyl (TNP)-coupled erythrocytes by using plaque assays (5, 6) or spot tests (2). IgM was biosynthetically labeled with [^{35}S]methionine and was purified by binding to 2,4-dinitrophenyl-Sepharose, and the μ and κ chains were visualized by Na-DodSO$_4$/PAGE after reduction of disulfide bonds as described (7). The concentration of wild-type IgM in culture supernatants was measured by an ELISA specific for the fourth domain of C_μ ($C_{\mu 4}$) (8, 9).

RESULTS

Detection of Homologous Recombination. The system that we have used to detect homologous recombination is based on the hybridoma Sp6, which bears a single copy of the μ gene (10) and makes IgM (κ chain) specific for the hapten TNP (2). The mutant hybridoma cell line igm482 was derived from Sp6 and bears a 2-base-pair (bp) deletion in the exon encoding $C_{\mu 3}$, the third domain of C_μ, and therefore produces a truncated μ chain lacking the $C_{\mu 4}$ domain (11). IgM bearing this mutant μ chain does not activate complement, which provides a convenient assay for distinguishing mutant and wild-type cells; that is, Sp6 cells make plaques (efficiency = 0.5) on TNP-coupled erythrocytes, whereas igm482 cells do not (efficiency $< 10^{-7}$). The 2-bp deletion destroys an *Xmn* I restriction site in the $C_{\mu 3}$ exon, a feature that can be used to test for the restoration of the wild-type nucleotide sequence.

To make the transfer vectors pRCμ and pTCμ (Fig. 1), a 4.3-kilobase (kb) DNA segment encoding only the C_μ region was inserted in both orientations in pSV2neo, which confers resistance to the antibiotic G418 (13). Because these vectors do not include DNA for the heavy-chain variable region, they cannot themselves encode a μ heavy chain. However, if the vectors integrate into the chromosomal DNA of igm482 so as to juxtapose the normal C_μ region of the vector and the chromosomal TNP-specific heavy-chain variable region, the

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Abbreviations: C_μ region, μ-chain constant region; PFC, plaque-forming cells; TNP, 2,4,6-trinitrophenyl; G418^R, G418 resistant (resistance).

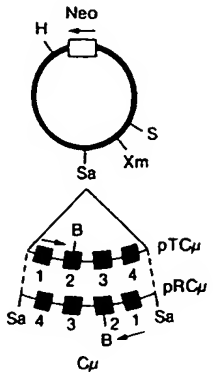

FIG. 1. Construction of transfer vectors. The segment of the μ gene bearing the exons (filled boxes) for the four domains of the C_μ region was inserted in both orientations in a derivative of pSV2neo that lacks the *Bam*HI site (12). For the construction of pRCμ and pTCμ, the *Eco*RI site of pSV2neo was converted to a *Sal* I site. The 4.3-kb *Xba* I fragment encoding the C_μ region was inserted in the modified pSV2neo by using *Xba* I/*Sal* I adapters generating 10-kb vectors in which the transcriptional orientation of the C_μ region is in tandem (pTCμ) or reversed (pRCμ) to the *neo* gene. These vectors have neither *Eco*RI nor *Xba* I sites. The unique *Bam*HI site in the $C_{\mu 2}$ exon of the insert was used to linearize the vector for electroporation. H, *Hpa* I; S, *Sca* I; Xm, *Xmn* I; Sa, *Sal* I; B, *Bam*HI.

resulting recombinant DNA will then encode a normal TNP-specific μ chain. We have assayed for homologous recombination of this type by measuring the frequency of PFC after transfer of these vectors to the igm482 cells (Table 1). The frequency of PFC among untreated igm482 cells is $<10^{-7}$. The frequency of PFC is at least 3- to 5-fold higher among cells that have been electroporated with pRCμ or pTCμ. In the G418-resistant ($G418^R$) populations, the frequency of PFC has increased to $\approx 10^{-3}$. Oligonucleotide-directed mutagenesis (12, 14) was used to introduce the 2-bp deletion present in the μ gene of igm482 into the pRCμ vector, thus generating the vector pRCμ482. The pRCμ482 vector generates $G418^R$ transformants, but does not yield PFC (i.e., none was detected among 2.5×10^4 independent $G418^R$ transformants), implying that the capacity to generate PFC depends on the normal C_μ region of these vectors. As described in the legend to Table 1, we estimate that this gene transfer protocol yields approximately 3 independent PFC from 2×10^7 recipient igm482 cells.

Analysis of μ Protein and DNA Structure. To examine the structure of the μ gene and protein in the PFC, representative transformants were cloned by sib selection from independent DNA transfer experiments. Transformants of igm482 that were derived from pTCμ and pRCμ are designated by the prefixes Im/TCμ and Im/RCμ, respectively (Table 1). In addition, we isolated two randomly selected $G418^R$ transformants, which are designated by R/RCμ and R/TCμ.

We have applied two other tests to verify that the IgM made by the PFC transformants has normal structure. The μ chains of the wild-type and igm482 cell lines differ in molecular mass by ≈15 kDa and are readily distinguished by their mobility in $NaDodSO_4$/PAGE (Fig. 2). All of the Im/RCμ and Im/TCμ transformants produce full-length μ chains indistinguishable from wild-type μ and have apparently ceased to make the mutant μ chain. We have also used a $C_{\mu 4}$-specific ELISA to quantify IgM production and to verify that the TNP-specific wild-type IgM made by these transformants includes the $C_{\mu 4}$ domain (8, 9). Compared to the wild-type Sp6 cells, the Im/RCμ transformants produced approximately the same amount of normal IgM (0.5–1.3 times as much), whereas the Im/TCμ transformants produced approximately 4 times less (0.1–0.4 times as much).

These results indicate that the $G418^R$ plaque-forming transformants make cytolytic IgM bearing a full-length, $C_{\mu 4}$-positive μ chain and thus suggested that the transferred DNA in the Im/TCμ and Im/RCμ transformants had integrated such that the wild-type C_μ region was now adjacent to the TNP-specific heavy-chain variable region. To test this possibility, and in particular to test whether the vectors had integrated by homologous recombination, we measured the size of μ gene and vector fragments generated from transformant DNA by various restriction enzymes. Fig. 3 shows

Table 1. Restoration of normal IgM production by DNA transfer to igm482 cells

DNA transferred	Frequency of $G418^R$ transformants	Frequency of PFC		PFC isolated for DNA analysis
		Nonselected recipient cell	$G418^R$ transformants	
None	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	—	—
pTCμ	8.3×10^{-4}	5×10^{-7}	$0.2–6 \times 10^{-3}$	Im/TCμ-27.2 (Im/TCμ-38.15) Im/TCμ-3.13 (Im/TCμ-3.10)
pRCμ	9.5×10^{-4}	3×10^{-7}	$1–12 \times 10^{-3}$	Im/RCμ-5C3 Im/RCμ-2A2 Im/RCμ-D7 (Im/RCμ-G3)
pRCμ482	1.3×10^{-3}	$<1 \times 10^{-7}$	$<0.04 \times 10^{-3}$	—

The indicated DNAs (50 μg) were linearized as described in Fig. 1, mixed in PBS (2) with 2×10^7 igm482 cells, and subjected to a 25-μF, 700-V pulse. Cell survival averaged 22%. After incubation in DMEM (2, 3) for 3 or 4 days, cells were assayed for PFC (5) on TNP-coupled erythrocytes and were distributed at limiting dilution in microtiter wells in DMEM containing G418 at 0.6 mg/ml to measure the frequency of $G418^R$ transformants. The $G418^R$ cells were selected in batch culture and tested for PFC. Repeated transfer of pRCμ482 DNA to igm482 generated a total of 2.5×10^4 independent $G418^R$ transformants but no PFC (i.e., the frequency of PFC is less than 0.04×10^{-3} per $G418^R$ transformant). Reconstruction experiments indicated that wild-type (Sp6) cells yield detectable plaques with the same efficiency (≈0.5) in the absence and presence of up to 10^7 igm482 cells. The plaque-forming transformants were isolated by sib selection from the indicated gene transfer experiments. Transformants listed in parentheses are not necessarily independent of the one listed above, as they derive from the same gene transfer experiment. We can estimate the number (N) of independent PFC that these gene transfer protocols generate, subject to two assumptions—namely, that the plaquing efficiency of the PFC is 0.5, like the normal cells and that the PFC arise immediately after electroporation. Then $N = (2 \times 10^7) \times$ (fraction of surviving cells) × (frequency of PFC in nonselected recipient cells) × (1/0.5) ≈ 3.

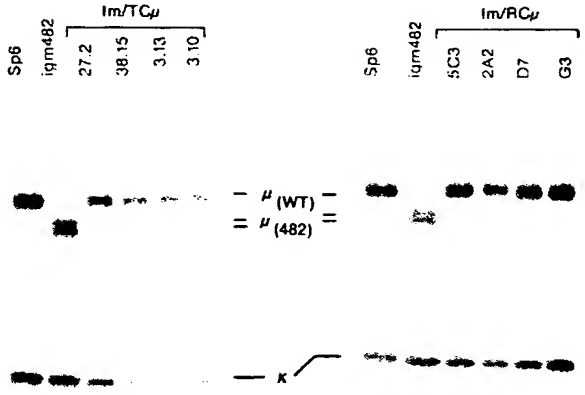

FIG. 2. Analysis of μ chains produced by plaque-forming transformants. The indicated cell lines were incubated in medium containing [^{35}S]methionine to biosynthetically label protein. IgM was purified by binding to 2,4-dinitrophenyl-Sepharose. The μ and κ chains were analyzed by NaDodSO$_4$/PAGE after reduction of disulfide bonds (7). We have found that μ chains made by igm482 in the presence of tunicamycin migrate as a single band (data not shown), suggesting that the double band seen here reflects alternative modes of glycosylation.

fragment sizes expected from the μ genes of igm482 and from the transformant DNA if the transfer vectors have integrated by homologous recombination. The μ genes of igm482 and Sp6 are identical except that the *Xmn* I site present in the $C_{\mu 3}$ exon of Sp6 is destroyed by the 2-bp deletion of igm482. The origins of the probes are shown in Fig. 3*A*: probes a, b, and e contain sequences that are present in the chromosomal μ gene but not in the transfer vectors; probe f contains sequences present in both the chromosomal and vector DNAs; and probe g is the 762-bp *Pvu* II fragment from the *neo* gene. Some of the μ-gene probes detect other (joining region or cross hybridizing) sequences in the hybridoma DNA. To indicate these irrelevant bands, we have included the cell line igm10, an Sp6-derived mutant cell line that has been shown to lack the TNP-specific μ gene (10).

Fig. 4 *A* and *B* present a *Sca* I digest analyzed with probes b and g, respectively. These results indicate that for all the Im/TCμ and Im/RCμ transformants, except Im/RCμ-5C3, the observed fragment sizes correspond with expectations for the case where a single copy of the vector has integrated into the chromosomal μ gene by homologous recombination. The bands detected for DNA from Im/RCμ-5C3 by probe b have the same mobility as for igm482. The low ($<10^{-7}$) frequency of PFC in the igm482 population argues that this transformant did not arise by reversion of the frameshift mutation and consequently suggests that the μ gene was in this case restored by a double crossover or gene conversion event, with the simultaneous acquisition of G418^R by an independent integration event. The same conclusions as to the structure of the recombinant μ genes in the plaque-forming transformants is supported by the analysis of *Eco*RI-digested DNA with probe b (Fig. 4*C*) and *Hpa* I-digested DNA with probes b and g (data not shown). The cell lines R/RCμ-1, R/RCμ-2, R/TCμ-1, and R/TCμ-2 were selected as G418^R transformants and do not produce normal IgM. They, as well as the transformant Im/RCμ-5C3, are expected to have integrated the transfer vector at more-or-less random chromosomal sites. As expected, the sizes of the bands differ among these transformants (Fig. 4*B*).

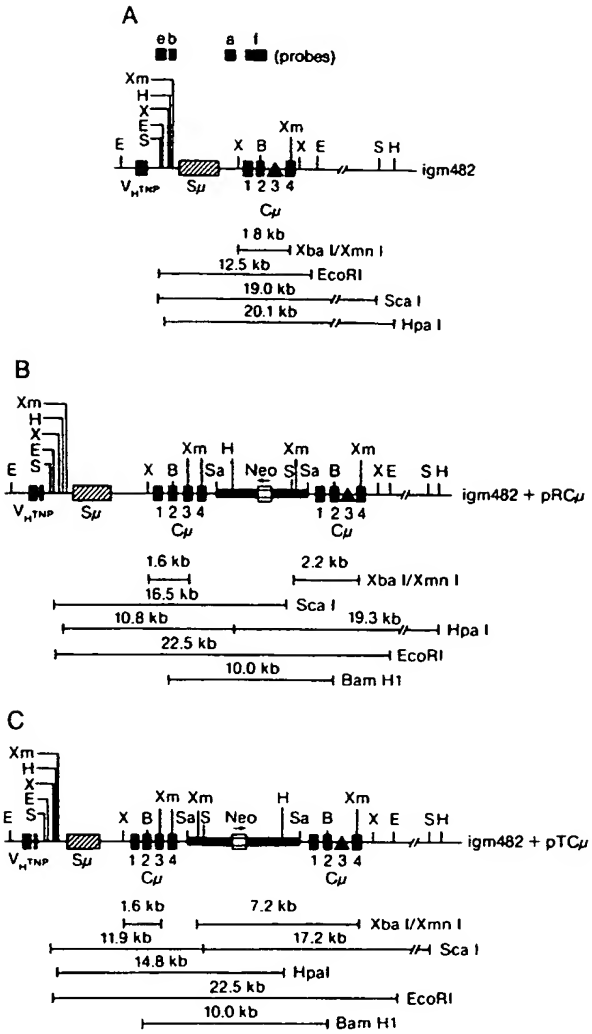

FIG. 3. DNA fragments predicted for the transformant μ genes. The sizes (in kb) that the indicated restriction enzymes should generate are shown for the μ gene of igm482 (*A*) and for transformants where a single copy of the pRCμ (*B*) or pTCμ (*C*) vector has integrated by homologous recombination into the chromosomal igm482 μ gene so as to restore the wild-type μ sequence (from ref. 15; F. Blattner, personal communication). Probe a is the 968-bp *Hin*dIII/*Xba* I fragment; probe b is the 915-bp *Sst* I fragment; probe e is a 1983-bp *Bam*HI/*Eco*RI fragment; probe f is an 870-bp *Xba* I/*Bam*HI fragment; and probe g is the 762-bp *Pvu* II fragment from the *neo* gene of pSV2neo (13). E, *Eco*RI; S, *Sca* I; X, *Xba* I; H, *Hpa* I; Xm, *Xmn* I; B, *Bam*HI; Sa, *Sal* I; V$_H$TNP, TNP-specific heavy-chain variable region.

The pTCμ and pRCμ transfer vectors were linearized before electroporation by digestion at the unique *Bam*HI site in the $C_{\mu 2}$ exon. The transformants that have integrated a vector into the chromosomal μ gene yield a 10-kb *neo*-containing *Bam*HI fragment (data not shown). As shown in Fig. 3, this result indicates that both copies of the C_μ region have their *Bam*HI site intact.

The 2-bp deletion of igm482 destroyed an *Xmn* I site, which would be restored if the vectors integrate by homologous recombination (Fig. 3). By using probe f on DNA that has been digested with a combination of *Xba* I and *Xmn* I, we therefore expected to detect fragments of 1.8-kb from the mutant μ gene compared to 1.6-kb from the wild-type. In addition, the integration of the pTCμ and pRCμ vectors generates downstream fragments that will be detected with

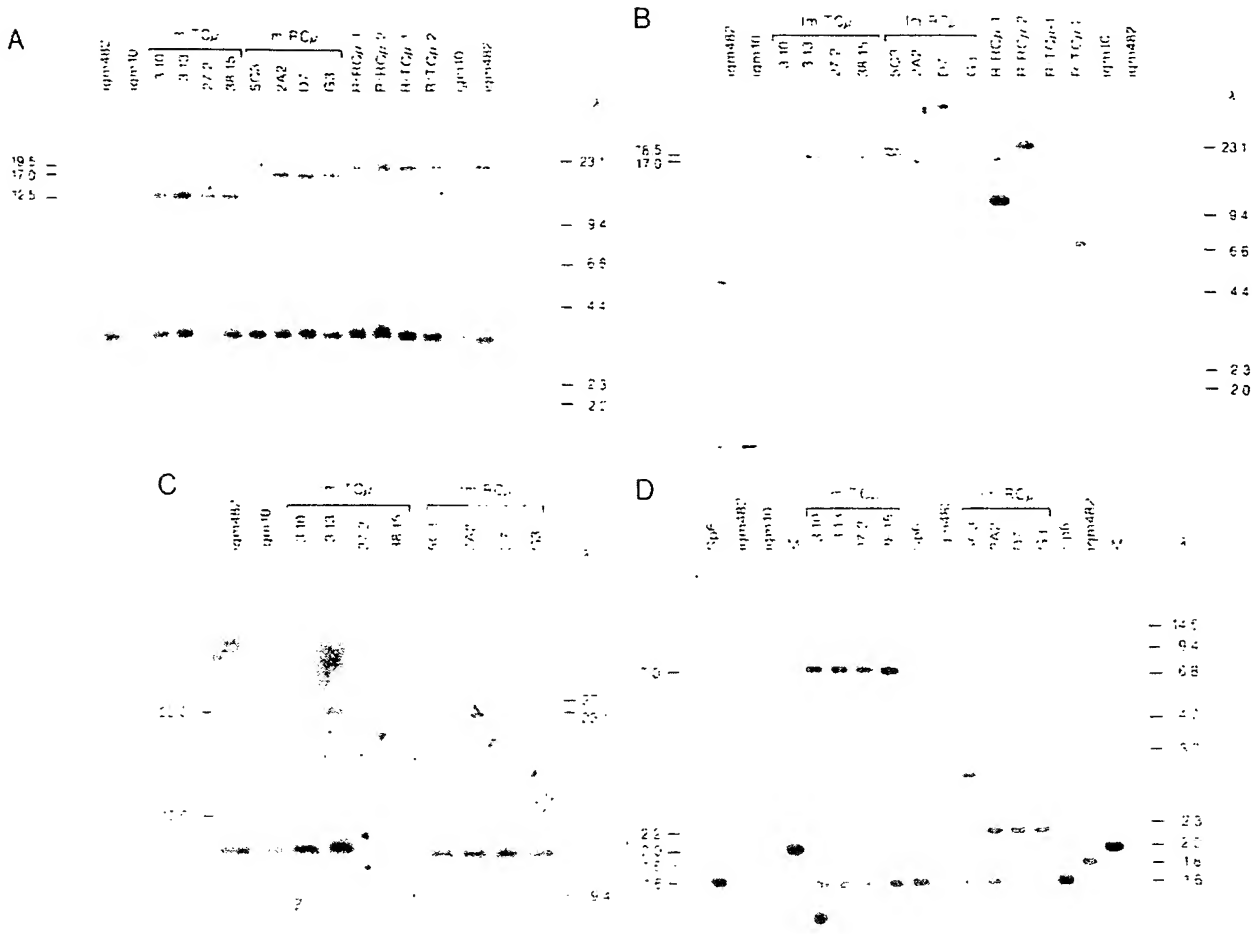

FIG. 4. Analysis of transformant DNA structure. DNA from the indicated cell lines was digested with *Sca* I (*A* and *B*), *Eco*RI (*C*), or *Xba* I/*Xmn* I (*D*) and was probed with probe b (*A* and *C*), probe g (*B*), or probe f (*D*). The size of each band (in kb) of interest is indicated to the left of the blot and was calculated by comparison with the position of the λ marker bands indicated to the right of the blot. M, position of 2.0-kb marker.

this probe. If these fragments have retained the 2-bp deletion of igm482, they are expected to be 7.2 and 2.2 kb from the Im/TCμ and Im/RCμ transformants, respectively, whereas they will be 7.0 and 2.0 kb if they have the wild-type sequence. As shown in Fig. 4*D*, all the Im/RCμ and Im/TCμ transformants show the 1.6-kb band, as expected for transformants that have acquired the wild-type μ gene sequence. The Im/RCμ transformants 2A2, G3, and D7 also show a 2.2-kb band clearly resolved from the 2.0-kb marker (lane M), implying that the downstream copy of the μ gene has retained the 2-bp deletion. The recombinant Im/RCμ-5C3, which is presumably a case of gene replacement, shows a 3-kb fragment in addition to the 1.6-kb band. We suppose that this band derives from the vector integration event that conferred $G418^R$. In addition to the 1.6-kb band, the Im/TCμ transformants also yield a fragment migrating at ≈7 kb. This gel did not resolve 7.2 kb from 7.0 kb and consequently does not indicate whether the downstream C_μ segment in these transformants has retained the 2-bp deletion.

In other digestions, we have confirmed that the chromosomal DNA segment bearing the TNP-specific heavy-chain variable region–C_μ intron has not been altered by the integration of the vectors. That is, by using probe e on *Eco*RI-digested DNA and probe a on *Xba* I- and *Xmn* I-digested DNA, we have shown that the transformant and Sp6 bands comigrate (data not shown). In summary, these results are in each case consistent with the hypothesis that the μ gene in the Im/RCμ and Im/TCμ transformants has been restored by homologous recombination with the transfer vector and that in the cases of integrative recombination the crossovers have occurred between the *Xba* I site just 5′ of $C_{\mu 1}$ and the *Xmn* I site in $C_{\mu 3}$.

DISCUSSION

We have shown that normal IgM production can be restored in the mutant hybridoma igm482 by the transfer of the pRCμ and pTCμ vectors, which encode a normal C_μ region, but not by the vector pRCμ482, which has the same 2-bp deletion as igm482 cells. We estimate that our standard gene transfer protocol, whereby 2×10^7 cells are electroporated in the presence of 50 μg of DNA, yields approximately three independent normal IgM-producing transformants, as detected by their plaque-forming capacity. By selecting for $G418^R$ transformants, the frequency of wild-type μ-gene recombinants is raised to $\approx 10^{-3}$. Other investigators have examined homologous recombination by using different genes, different methods of DNA transfer, and different requirements for gene replacement versus vector integration and have estimated that the ratio of homologous to nonhomologous integrative recombinations ranged from 10^{-2} to 10^{-3} (16–22).

We have concentrated, in this study, on the G418^R transformants because of the greater ease with which the PFC can be isolated and have analyzed five independent transformants of the frameshift mutant igm482, which were selected for their ability to produce normal IgM. Analysis of the μ-gene structure shows that in four of the five transformants, a single copy of the transfer vector has integrated into the chromosomal μ gene. One transformant, Im/RCμ-5C3, has acquired a wild-type μ gene but has not integrated the vector at that locus. We suppose that this transformant represents a case of gene replacement, perhaps by a double crossover or some mechanism of gene conversion. In such recombinants, G418^R must be acquired by a second vector integration event, implying that among the cells that have restored their μ gene by homologous recombination the ratio of gene replacement to vector integration should be lower among G418^R cells than in the unselected population. Inasmuch as we have estimated that our standard procedure yielded approximately three plaque-forming transformants, of which at least one occurred in each case by vector integration, we conclude that the frequency of G418-sensitive PFC in the unselected population is not enormously higher than G418^R PFC. The transformants that integrated the vector into the μ gene have acquired only one copy of the transferred DNA, which evidently is sufficient to confer G418^R. We do not know if the integration of multiple vectors in tandem in the μ gene would depress its expression, thus precluding the isolation of such transformants as PFC.

Thomas and Capecchi (20) have used a chromosomally integrated *neo* gene bearing an amber mutation as a target and have observed that transfer of a segment of wild-type *neo* DNA stimulates the generation of frameshift mutations that suppress the effects of the amber mutation. The igm482 cell line has a 2-bp deletion, and it seems likely that nearby frameshift mutations would suppress the igm482 mutation. However, we have found that in all five independent plaque-forming transformants the mutation of igm482 has been eliminated, suggesting that the stimulated generation of frameshift mutations might reflect peculiarities of the *neo* DNA sequence, such as were pointed out (20).

The Im/RCμ transformants produce, on the average, as much normal IgM as the wild-type hybridoma Sp6 and 4-fold more than the Im/TCμ transformants. The Im/RCμ and Im/TCμ transformants presumably differ only in the orientation of the integrated pSV2neo, thus suggesting that the vector can affect the expression of the nearby μ gene. The Im/RCμ and Im/TCμ transformants seem to be more uniform in their μ-gene expression than stable transformants that have integrated single or multiple copies of complete μ genes at different chromosomal sites and show variable and usually low expression (12, 23, 24). At least for the Im/RCμ transformants, this apparent uniformity of expression cannot be attributed to their having been selected for their plaque-forming capacity, as the (plaque-forming) Im/TCμ transformants make 4 times less IgM. The difference between the expression of the μ gene at its normal and other loci suggests the possibility that the cloned μ gene lacks as yet unidentified elements that are needed for the normal, high-level expression. Some of the hybridoma mutants that have been selected for low-level μ-gene expression (2–4) might be defective in such elements. The finding that homologous recombination can be used to correct and thus map chromosomal mutations in the μ gene suggests that this technology might be used to identify the mutations that affect μ-gene expression. Conversely it might be possible to extend this system to introduce well-defined mutations into the normal μ gene.

The integration of the vectors into the μ gene by homologous recombination has placed the vector-borne C_μ segment so as to separate the original chromosomal DNA segments for the variable and (mutant) C_μ regions. The NaDodSO$_4$/PAGE analysis (Fig. 2) indicates that these transformants have ceased to produce the mutant μ chain. It should, therefore, be possible to use this type of recombination as a much easier method of producing specifically modified immunoglobulin than is currently available. For example, immunoglobulin might be more effective in therapy if it had an altered constant region (e.g., of human rather than mouse origin or toxin linked). DNA encoding the appropriate constant region could be inserted in a transfer vector so as to lie 3′ of the major variable–constant (V–C) intron. Such vectors would be expected to integrate by homologous recombination so as to place the constant region of the transferred DNA in position to be expressed with the endogenous, chromosomal variable region, thus making it possible to produce optimized immunoglobulin without having to isolate, modify, and reexpress the endogenous variable-region genes.

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Exhibit C

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Patterns of Integration of DNA Microinjected into Cultured Mammalian Cells: Evidence for Homologous Recombination Between Injected Plasmid DNA Molecules

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We examined the fate of DNA microinjected into nuclei of cultured mammalian cells. The sequence composition and the physical form of the vector carrying the selectable gene affected the efficiency of DNA-mediated transformation. Introduction of sequences near the simian virus 40 origin of DNA replication or in the long terminal repeat of avian sarcoma provirus into a recombinant plasmid containing the herpes simplex virus thymidine kinase gene (pBR322/HSV-tk) enhanced the frequency of transformation of LMtk$^-$ and RAT-2tk$^-$ cells to the TK$^+$ phenotype 20- to 40-fold. In cells receiving injections of only a few plasmid DNA molecules, the transformation frequency was 40-fold higher after injection of linear molecules than after injection of supercoiled molecules. By controlling the number of gene copies injected into a recipient cell, we could obtain transformants containing a single copy or as many as 50 to 100 copies of the selectable gene. Multiple copies of the transforming gene were not scattered throughout the host genome but were integrated as a concatemer at one or a very few sites in the host chromosome. Independent transformants contained the donated genes in different chromosomes. The orientation of the gene copies within the concatemer was not random; rather, the copies were organized as tandem head-to-tail arrays. By analyzing transformants obtained by coinjecting two vectors which were identical except that in one a portion of the vector was inverted, we were able to conclude that the head-to-tail concatemers were generated predominantly by homologous recombination. Surprisingly, these head-to-tail concatemers were found in transformants obtained by injecting either supercoiled or linear plasmid DNA. Even though we demonstrated that cultured mammalian cells contain the enzymes for ligating two DNA molecules very efficiently irrespective of the sequences or topology at their ends, we found that even linear plasmid DNA was recruited into the concatemer by homologous recombination.

DNA-mediated gene transfer permits the introduction of new genetic information into cultured mammalian cells. This technique is useful for studying the factors which influence gene expression, studying the mechanism of somatic cell recombination, and isolating mammalian genes. In 1977, Bacchetti and Graham (3), Maitland and McDougall (23), and Wigler et al. (41) demonstrated that the herpes simplex virus thymidine kinase gene (HSV-tk gene) could be transferred into LMtk$^-$ cells, a mouse cell line deficient in thymidine kinase. The rare transformants that expressed thymidine kinase activity were isolated by growing the cells in HAT medium (36). Cellular uptake of the purified DNA was facilitated by the formation of a DNA-calcium phosphate coprecipitate (18). Coprecipitation of the selectable gene with carrier DNA was found to increase the transformation efficiency by two to three orders of magnitude. Although the role of the carrier DNA, which can be any vertebrate DNA, has not been fully elucidated, this DNA may provide sequences or DNA structures which enhance integration.

Nonselectable genes can also be introduced into cultured mammalian cells by cotransformation with unlinked but selectable genes (42). By this method virtually any purified gene can be introduced into mammalian cells. Perucho et al. (28) have shown that cotransformed sequences are linked in transformed cells such that loss of the selectable gene from the transformant is frequently accompanied by a concurrent loss of all cotransformed sequences. Perucho et al. also demonstrated that fragments of carrier DNA and DNA for selectable and nonselectable genes are randomly ligated together in the recipient cells to form a large concatenate, which can exceed

0.2% of the haploid genome of the host. The concatenate is integrated into one or a very few sites in the host chromosome to form a stable transformant (30, 32). In independent stable transformants the concatenate is integrated into different chromosomes (30). In contrast, in unstable transformants the concatenate may exist transiently as an independent replicating unit (31).

An alternative method for transferring purified genes into cultured mammalian cells is to inject DNA into nuclei with glass micropipettes (2, 9, 17). The conditions for gene transfer by microinjection and calcium phosphate coprecipitation differ in two important ways. Microinjections are done in the absence of carrier DNA, and the amount of DNA introduced can be controlled more precisely. It has been shown that in the absence of carrier DNA, the injection of only a few molecules per cell is sufficient to obtain transformants with a high efficiency (9). The molecular events that affect a few plasmid DNA molecules introduced into the nuclear environment may be quite different from the events faced by the same plasmid DNA molecules introduced as a calcium phosphate coprecipitate in a sea of carrier DNA.

In this study we examined the fate of DNA introduced into cultured mammalian cells by microinjection.

MATERIALS AND METHODS

The methods used for culturing cells, autoradiography, plasmid DNA preparation, and microinjection have been described elsewhere (9, 10).

Southern transfer analysis. Confluent plates of cells were rinsed with phosphate-buffered saline and lysed by adding 3 ml of lysis buffer (0.5% sodium dodecyl sulfate, 100 mM NaCl, 10 mM EDTA, 20 mM Tris, pH 7.6) to each 100-mm plate. Pronase was added to a final concentration of 0.2 mg/ml, and the lysates were incubated at 37°C for 1 to 2 h. The lysates were extracted with phenol-chloroform (1:1) and chloroform before precipitation with 2 volumes of ethanol. The DNA was washed with 70% ethanol, air dried, and suspended in 10 mM Tris (pH 7.4)–1 mM EDTA. Cellular DNAs were cleaved to completion with restriction enzymes according to the instructions of the manufacturers (New England Biolabs and Bethesda Research Laboratories). The extent of digestion was monitored by adding λ DNA as an internal standard. The cleaved cellular DNA (10 to 20 μg/well) was subjected to electrophoresis through neutral 0.6% agarose gels and transferred to nitrocellulose filters by the method of Southern (33). Plasmid sequences were detected by hybridization with the appropriate recombinant tk gene-containing plasmid labeled with ^{32}P by nick translation (24, 29) with [α-^{32}P]dCTP to a specific activity of $>10^8$ dpm/μg. After prehybridization, the filters were hybridized in 4× SET (150 mM NaCl, 2 mM EDTA, 30 mM Tris [pH 8]) containing 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, 20 μg of tRNA per ml, and 10× Denhardt solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrollidone) at a probe concentration of 10 ng/ml at 68°C for 24 to 48 h. The filters were washed stepwise in decreasing concentrations of SET buffer (3×, 1×, and 0.5× SET supplemented with 0.1% sodium dodecyl sulfate and 0.1% sodium pyrophosphate) at 68°C. The filters were then dried and exposed for 24 to 72 h at −70°C to Kodak XAR-5 film, using a Du Pont Lightning Plus intensifying screen.

In situ hybridization. Transformants were grown in T-75 flasks in Dulbecco modified Eagle medium containing HAT (0.1 mM hypoxanthine, 1.1×10^{-3} mM amethopterin, 1.65×10^{-2} mM thymidine) and 10% fetal calf serum. The cells were diluted 1:4 the night before metaphase spreads were prepared as described previously (40) from cells treated with 100 μg of colcemid per ml for 30 to 45 min at 37°C. Probe prepared by nick translation with [^3H]dTTP (>100 Ci/mol) was found to be equivalent to that prepared with [^{125}I]dCTP.

RESULTS

Parameters affecting the frequency of transformation. When plasmid DNA containing the HSV-tk gene is injected into the nuclei of LMtk$^-$ cells, some of the cells are transformed to the TK$^+$ phenotype (2, 9). The proportion of LMtk$^-$ cells that are transformed depends on the nature of the plasmid DNA into which the HSV-tk gene is inserted (9) (Table 1). Approximately 1 LMtk$^-$ cell in 100 receiving a nuclear injection of plasmid pBR322/HSV-tk was transformed to TK$^+$ (Table 1). The transformation frequency increased to more than one in five when the injected HSV-tk gene-containing plasmid also contained sequences near the simian virus 40 (SV40) origin of DNA replication (*ori*) or sequences in the long terminal repeat (LTR) of avian sarcoma virus (ASV) provirus (Fig. 1 shows recombinant plasmid construction). It is interesting that placing these SV40 or ASV "enhancer" sequences either 5′ or 3′ to the HSV-tk gene resulted in the same relative increase in transformation frequency (Table 1). The phenomenon of enhancement of DNA-mediated transformation with respect to the location and function of enhancer sequences was explored in a separate communication (P. A. Lucius, J. M. Bishop, H. E. Varmus, and M. R. Capecchi, submitted for publication).

The effect of enhancer sequences on the transformation frequency was not restricted to LMtk$^-$ cells since a comparable increase in transformation frequency was mediated by the same enhancer sequences in RAT-2tk$^-$ cells (Table 1). Interestingly, the DNA-mediated transformation efficiency of RAT-2tk$^-$ cells was consistently lower than that of LMtk$^-$ cells, even though the viability and the ability to express the HSV-tk gene transiently are nearly 100% for both cell lines.

Another factor which influences transformation efficiency is the physical form of the inject-

TABLE 1. Transformation frequencies obtained by injecting HSV-tk gene-containing plasmid DNAs[a]

Cell line	DNA injected	No. of transformants per 10^3 cells receiving an injection
LMtk$^-$	pBR322/tk	14
	pBR322/SV-0/tk A	310
	pBR322/SV-0/tk B	262
	pBR322/ASV-2LTR/tk A	220
	pBR322/ASV-2LTR/tk B	214
RAT-2tk$^-$	pBR322/tk	1
	pBR322/SV-0/tk A	48
	pBR322/SV-0/tk B	36
	pBR322/ASV-2LTR/tk A	30
	pBR322/ASV-2LTR/tk B	28

[a] LMtk$^-$ and RAT-2tk$^-$ cells were grown on cover slips (10 by 10 mm) in 35-mm petri dishes. From 1 to 50 cells per dish received nuclear injections with the DNA solutions. The number of cells per dish receiving an injection was chosen such that at the conclusion of the experiment many of the petri dishes contained no transformed colonies. After the injections the cells were incubated for 24 h in nonselective medium at 37°C in a 5% CO_2 incubator and then switched to HAT medium. After 2 weeks the dishes were scored for the presence of large, healthy colonies. We previously reported that 1 LMtk$^-$ cell in 500 receiving an injection of pBR322/HSV-tk DNA was transformed to the TK$^+$ phenotype. The higher transformation frequencies observed were attributed to improved microinjection procedures. RAT-2tk$^-$ cells were obtained from W. Topp (38).

ed plasmid DNA. As Fig. 2 shows, comparable transformation frequencies were observed by injecting more than 50 molecules of either linear or supercoiled pBR322/SV-0/tk DNA per cell. However, as the number of plasmid DNA molecules injected per cell was decreased to less than 50, a marked difference in transformation efficiency appeared. A high transformation efficiency (approximately 20%) was retained by injecting an average of as few as five linear molecules per cell, whereas the transformation frequency dropped to less than 1 in 200 when 5 to 10 supercoiled molecules were injected per cell.

The observed differences in the transforming efficiencies of linear and supercoiled molecules were not restricted to transformation of LMtk$^-$ cells with HSV-tk gene-containing vectors. Similar results were obtained when linear and supercoiled HSV-tk gene-containing plasmids were injected into RAT-2tk$^-$ cells. Moreover, we found that linear plasmids containing the selectable *Escherichia coli* gene for xanthine guanine phosphoribosyl transferase (27) gave a 30- to 50-fold-higher transformation frequency than supercoiled plasmids, when less than 10 copies per cell were injected into LMtk$^-$ or RAT-2tk$^-$ cells.

Transformants containing a single copy of the HSV-tk gene. Transformants obtained after injecting only a few plasmid molecules per cell often contained a single copy of the transforming sequence. Figure 3 shows a Southern genomic transfer analysis of transformants obtained by injecting a few copies of either supercoiled or linear plasmid DNA. Both the intensity and the patterns of hybridization signals were consistent with the conclusion that these transformants contained single copies of their respective plasmid DNAs. When genomic DNA was incubated with a restriction endonuclease that did not cleave the appropriate plasmid DNA or cleaved it only once, the respective patterns consisted of a band that migrated more slowly than intact plasmid DNA or two bands of variable size. The diversity of band sizes generated by the restriction endonucleases suggests that the transforming DNA was flanked by different nonplasmid sequences in each transformant. Using a combination of appropriate restriction enzymes, only some of which are shown in Fig. 3, we constructed a restriction map of how each plasmid molecule was integrated into host DNA sequences (Fig. 3). From such analyses there did not appear to be a preferred site of integration within the supercoiled plasmids. For example, in the three transformants shown in Fig. 3, which were obtained by injecting pBR322/SV-0/tk B or pBR322/ASV-2LTR/tk A or B supercoiled DNA, two of the plasmids were integrated into host DNA through their pBR322 sequences and the third was integrated through its ASV-2LTR leader sequence.

On the other hand, single linear molecules appeared to be inserted into the host genome through their ends (Fig. 3, lanes j through m). In the process of insertion, the restriction sites at the ends of the plasmid molecule was lost (Fig. 3, lane j). However, extensive modification at the ends of the linear molecules did not appear to be the rule. For example, in transformants obtained by injecting pBR322/ASV-2LTR/tk A linearized with *Hin*dIII, the *Cla*I site which was only 6 nucleotide base pairs away from the *Hin*dIII site was still present (Fig. 3, lane l).

Transformants containing multiple copies of the HSV-tk gene. When the recipient cells received injections of more than 5 linear or 25 supercoiled HSV-tk gene-containing plasmid DNA molecules per cell, the resulting transformants contained multiple copies of the transforming DNA. Figure 4, lanes a through c show Southern genomic transfers of independent transformants obtained by injecting approximately 25 copies of pBR322/ASV-2LTR/tk B supercoiled DNA per cell into LMtk$^-$ cells. The genomic DNA was hydrolyzed with *Bgl*II, which cut the plasmid DNA once. In these

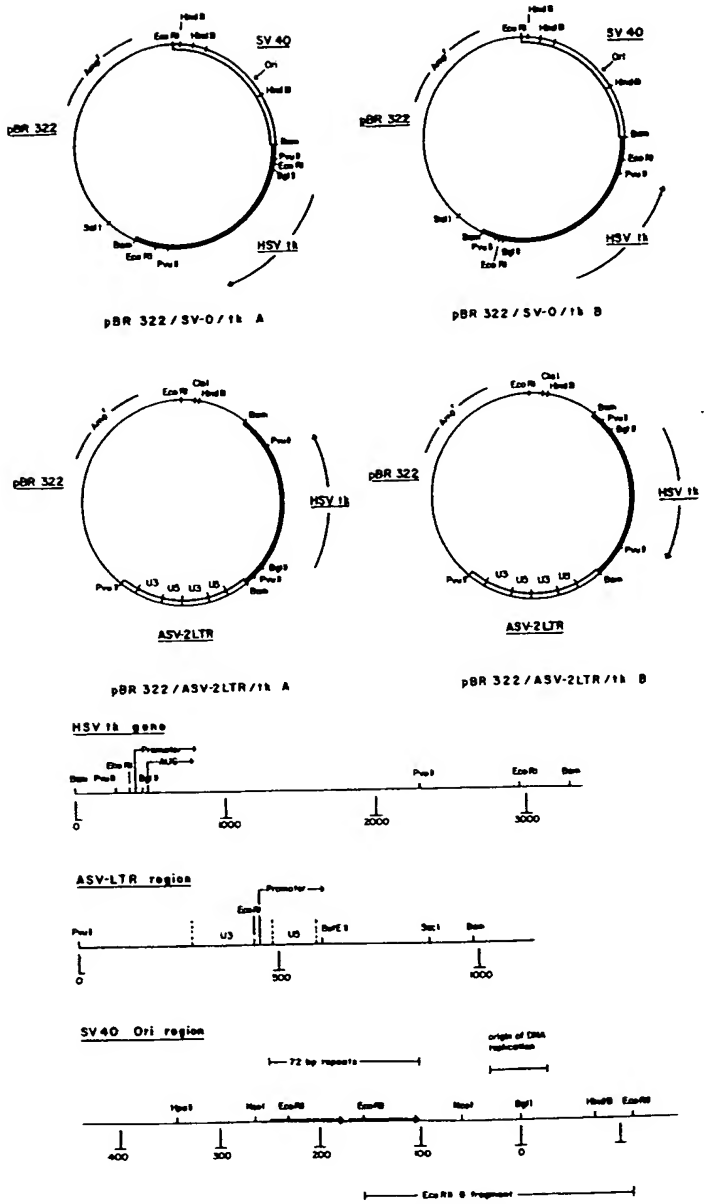

FIG. 1. Restriction maps of recombinant plasmids pBR322/SV-0/tk A, pBR322/SV-0/tk B, pBR322/ASV-2LTR/tk A, and pBR322/ASV-2LTR/tk B, as well as schematic representations of the HSV-tk gene-containing *Bam*HI (Bam) fragment, ASV LTR region, and SV40 origin of DNA replication (Ori) region. The construction of pBR322/SV-0/tk A and B recombinant plasmids has been described previously (9). pBR322/ASV-2LTR/tk A and B (obtained from and prepared by P. Luciw) were prepared by inserting the *Pvu*II fragment containing two copies of the u3u5-terminal repeats from the Schmidt-Ruppin A-2 strain of ASV DNA into pBR322 at the *Pvu*II site (14). The recombinant plasmid described above was then hydrolyzed with *Bam*HI, and the 3.4-kb *Bam*HI fragment containing the HSV-tk gene was inserted. In the process of inserting the HSV-tk gene-containing *Bam*HI fragment, 320 bp from the ASV leader sequence and 1.8 kb of the pBR322 sequence were removed. Amp^r, Ampicillin resistance.

transformants, we observed a single major band that hybridized with a ^{32}P-labeled nick-translated pBR322/ASV-2LTR/tk B probe and comigrated with intact linear pBR322/ASV-2LTR/tk B plasmid DNA. The intensity of this band relative to bands in single-copy transformants was consistent with the conclusion that these transformants contained 10 to 20 copies of the plasmid sequences. We often observed two other bands, which hybridized less intensely and the sizes of

which varied from transformant to transformant. These minor bands probably represented the fragments which included the junction between the concatemer and host sequences. When DNAs from the transformants described above were digested with an endonuclease that did not cleave the plasmid DNA, hybridization occurred over a single band that migrated much more slowly than linear plasmid DNA (data not shown). The above-described results are only consistent with the conclusion that the multiple plasmid sequences were present in the transformants as head-to-tail concatemers, since a random arrangement of molecules within the concatemers would have yielded multiple bands after cleavage with *Bgl*II.

In Fig. 4, lanes d through g, we show a series of LMtk$^-$ and RAT-2tk$^-$ transformants obtained by injecting approximately 25 copies of pBR322/ASV-2LTR/tk A DNA linearized with *Hin*dIII per cell. The patterns shown by a Southern transfer analysis of the genomic DNAs from these transformants digested with *Bgl*II were indistinguishable from the patterns obtained by injecting pBR322/ASV-2LTR/tk A or B supercoiled molecules. This result was surprising and indicated that the multiple copies of the HSV-tk gene-containing plasmid injected as linear molecules were incorporated into head-to-tail concatemers. To illustrate the expected hybridization pattern if the multiple gene copies within the concatemer were randomly oriented, pBR322/ASV-2LTR/tk A DNA was linearized with *Hin*dIII, ligated in vitro to form a random concatemer, digested with *Bgl*II, and electrophoresed in parallel with the genomic DNA digests. Three bands, corresponding to adjacent DNA molecules that were linked in the head-to-head (8.9 kilobases [kb]), head-to-tail (7.4 kb), and tail-to-tail (6.0 kb) configurations, were observed (Fig. 4, lane h). In some of the transformants obtained by injecting linear molecules, a hint of the bands predicted for adjacent molecules that were oriented head to head or tail to tail was observed. However, the relative intensities of these bands were not what would be predicted for a random concatemer (1:2:1 for the head-to-head, head-to-tail, and tail-to-tail configurations, respectively).

Genomic DNAs from several of these transformants were hydrolyzed with *Hin*dIII and analyzed as described above to determine how often the *Hin*dIII site was preserved during the incorporation of the linear plasmid DNA into the head-to-tail concatemer. Loss of a single nucleotide base pair at the ends during concatemer formation would have resulted in the loss of the *Hin*dIII site. This loss was revealed on the Southern transfer as a band that comigrated with a linear plasmid dimer or higher multimer. More than 90% of the *Hin*dIII sites were preserved

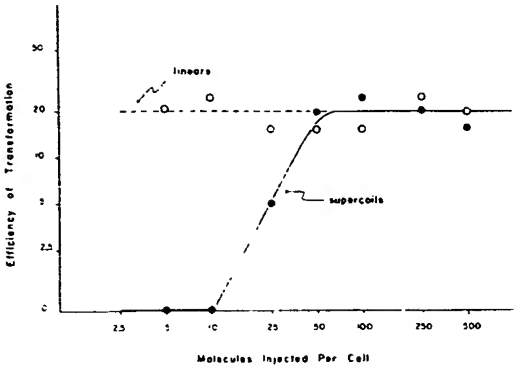

FIG. 2. Comparison of the frequency of transforming LMtk$^-$ to the TK$^+$ phenotype after nuclear injections of increasing amounts of linear (○) and supercoiled (●) pBR322/SV-0/tk B DNAs. The experimental procedure was as described in Table 1, footnote *a*. pBR322/SV-0/tk B plasmid DNA was linearized with *Sal*I.

during concatemer formation, indicating that the plasmid DNA underwent very little damage at the ends of the linear molecule before or during concatemer formation (data not shown; however, a similar conclusion can be reached from the experiments shown in Fig. 7, lanes i through l).

Head-to-tail concatemers are integrated into the host chromosome. Several transformants were examined by hybridization in situ to determine whether the transforming sequences were indeed integrated into host chromosomes. Two of the transformants chosen for this study, L11 and L278, were shown by a Southern transfer analysis to contain approximately 10 copies of pBR322/SV-0/tk B and 30 copies of pBR322/ASV-2LTR/tk B, respectively, as head-to-tail concatemers. L11 and L278 revealed a single pair and two pairs of junction fragments, respectively, which is consistent with integration of these concatemers at one and two sites in the host genome, respectively. Figure 5 shows metaphase spreads of L11 and L278 hybridized in situ to HSV-tk gene-containing recombinant plasmid DNA labeled by nick translation with [^{125}I]dCTP or [^{3}H]dTTP. The transforming DNA in L11 could be localized to a single chromosome, whereas in L278 the plasmid sequences appeared to have been integrated into two sites. Hybridization of the plasmid probe to parental LMtk$^-$ chromosomes showed no labeling (data not shown). The site of integration was different for other transformants examined.

How are the head-to-tail concatemers generated? Figure 6 shows three models by which head-to-tail concatemers could be generated. In model I a single plasmid sequence is first integrated into host DNA and then amplified, generating a head-to-tail concatemer. In models II and III the

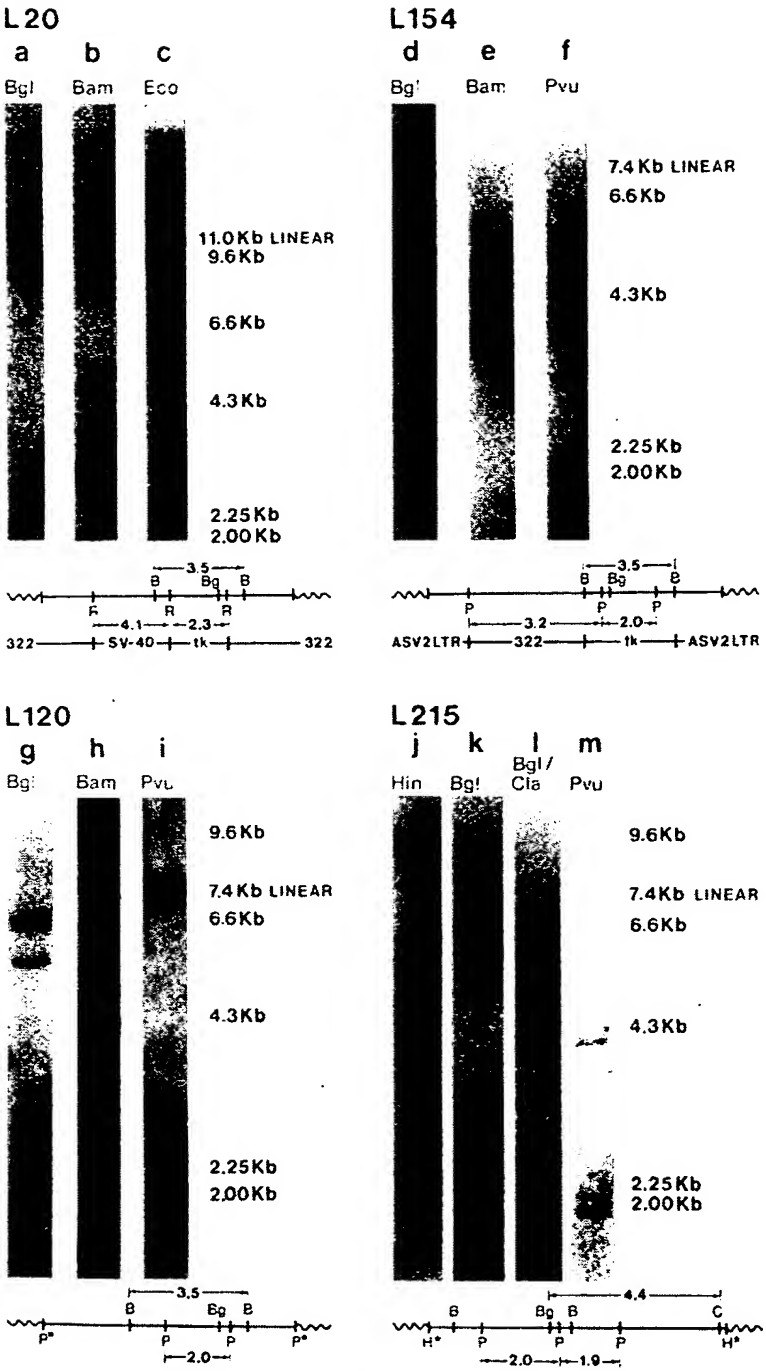

FIG. 3. Southern transfer analysis of LMtk$^-$ transformants containing single copies of the HSV-tk gene-containing vectors. Transformants L20 (lanes a through c), L154 (lanes d through f), and L120 (lanes g through i) were obtained by injecting LMtk$^-$ cells with approximately 10 copies of supercoils of pBR322/SV-0/tk B, pBR322/ASV-2LTR/tk B, and pBR322/ASV-2LTR/tk A DNAs per cell, respectively. L215 (lanes j through m) was obtained by injecting LMtk$^-$ cells with less than five copies of pBR322/ASV-2LTR/tk A DNA linearized with *Hin*dIII per cell. The restriction maps of the HSV-tk gene-containing vectors inserted into host DNA sequences were deduced from the Southern transfer data in lanes a through m. These maps were also consistent with a Southern transfer analysis of genomic DNAs digested with *Bam*HI, *Cla*I, *Hin*dIII, *Sal*I, *Cla*I plus *Bgl*II, *Cla*I plus *Bam*HI, and *Cla*I plus *Bgl*II plus *Bam*HI (data not shown). B and Bam, *Bam*HI; Bg and Bgl, *Bgl*II; C and Cla, *Cla*I; H and Hin, *Hin*dIII; P and Pvu, *Pvu*II; R and Eco, *Eco*RI. H* and P* indicate that the *Hin*dIII and *Pvu*II plasmid restriction sites were lost as a result of the plasmid DNA being inserted into the host genome. The extra band in the *Eco*RI digest of L20 (lane c) was a result of partial cleavage of the genomic DNA. In the *Bgl*II digest of L215 there are two bands, at 6.6 and 6.0 kb; in the *Bgl*II-*Cla*I digest there is a single band, at 4.4 kb. This indicates that a *Cla*I site is present in the flanking host DNA approximately 4.4 kb upstream from the *Bgl*II site and that the 4.4-kb band is a doublet. Consistent with this interpretation, we observed a band of approximately 9 kb when L215 DNA was cleaved with *Cla*I alone.

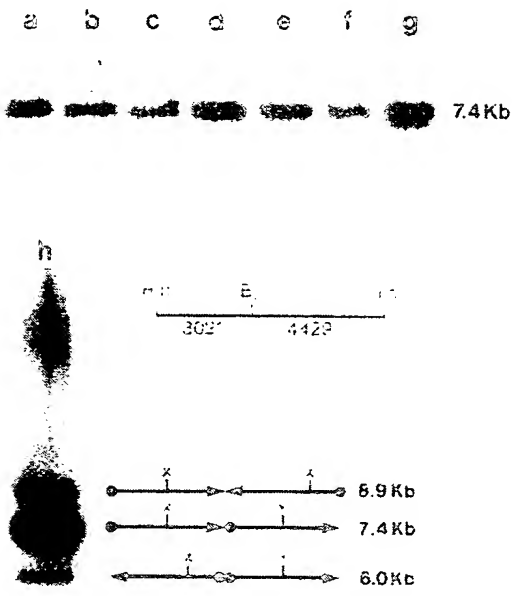

FIG. 4. Southern transfer analysis of LMtk$^-$ and RAT-2tk$^-$ transformants containing multiple copies of pBR322/ASV-2LTR/tk A or B DNA. Transformants L161, L162, and L163 (lanes a through c, respectively) were obtained by injecting LMtk$^-$ cells with approximately 25 copies of pBR322/ASV-2LTR/tk B supercoiled DNA per cell. Transformants L211, L212, R211, and R221 (lanes d through g, respectively) were obtained by injecting LMtk$^-$ and RAT-2tk$^-$ cells with approximately 25 copies of pBR322/ASV-2LTR/tk A DNA linearized with *Hin*dIII per cell. The transformants described above contained from 10 to 25 copies of plasmid DNA per cell. Genomic DNA from each transformant was incubated with *Bgl*II, which cut pBR322/ASV-2LTR/tk A or B once. The major band that hybridized with ^{32}P-labeled nick-translated pBR322/ASV-2LTR/tk A or B sequences comigrated with intact linear pBR322/ASV-2LTR/tk A or B plasmid DNA (7.4 kb). In the Southern transfers we did not detect junction fragments, presumably because these fragments were either too large or too small to allow detection under our transfer conditions. We did detect junction fragments in some of the transformants after cleavage of the genomic DNA with *Bam*HI. Lane h shows the Southern transfer pattern obtained by hydrolyzing with *Bgl*II a concatemer synthesized in vitro in which the pBR322/ASV-2LTR/tk A sequences were randomly oriented (see text). Hin, *Hin*dIII; Bgl, *Bgl*II.

head-to-tail concatemers are generated by a mechanism involving homologous recombination. In model II a single plasmid molecule is integrated into the host chromosome, perhaps by a nonhomologous recombination event, and subsequent plasmid molecules then integrate at the same site by homologous recombination. In model III the head-to-tail concatemer is generated by homologous recombination before its integration as a unit into a host chromosome.

To distinguish among the various models, we coinjected two HSV-tk gene-containing plasmids, A and B, which were identical except that the HSV-tk genes of the plasmids were in opposite orientations with respect to the pBR322 and ASV-2LTR sequences (Fig. 1). If the head-to-tail concatemer was generated by sequence duplication, then the neighbor of a given plasmid should always have been the same plasmid. The homologous recombination models predicted that the A and B vectors should be randomly interspersed. The arrangement of vectors within the head-to-tail concatemer could be identified by hydrolyzing genomic DNA with *Bgl*II, a restriction endonuclease that cleaves asymmetrically within the inverted HSV-tk gene sequence. If the A vectors were next to A vectors and the B vectors were next to B vectors, then the resulting plasmid DNA fragments from the concatemer should have been of unit length (7.4 kb). If the A and B vectors were interspersed, then two additional fragments that hybridized to the vector DNA were predicted, one longer (9.3 kb) and the other shorter (5.6 kb) than unit length (Fig. 7, lane h). Making the reasonable assumptions that (i) under conditions of equal input the probabilities of an A vector recombining with an A vector and with a B vector were the same and (ii) recombination occurred randomly throughout the plasmids, we predicted that the ratio of the intensities of the 9.3-, 7.4-, and 5.6-kb bands would be 1:6:1.

Figure 7, lanes a through c, show Southern transfer analyses of three transformants obtained by injecting LMtk$^-$ cells with equal numbers of pBR322/ASV-2LTR/tk A and B supercoiled molecules. The presence of the 9.3- and 5.6-kb hybridizing bands demonstrated that the A and B plasmid DNA molecules were interspersed within the concatemers, which is consistent with the homologous recombination models. Similar hybridization patterns were observed with transformants generated by coinjecting equal numbers of linear A and B molecules (Fig. 7, lanes d through g). As a reference for random end-to-end ligation, the A and B vectors were linearized with *Hin*dIII, mixed, ligated in vitro, and hydrolyzed with *Bgl*II. The more complex but predictable hybridization pattern obtained is shown in Fig. 7, lane h. Our results indicate that even with linear molecules, the concatemers were formed predominantly by homologous recombination rather than by random end-to-end ligation or amplification.

Deviation of the relative intensities of the 9.3-, 7.4-, and 5.6-kb bands in some of the transfor-

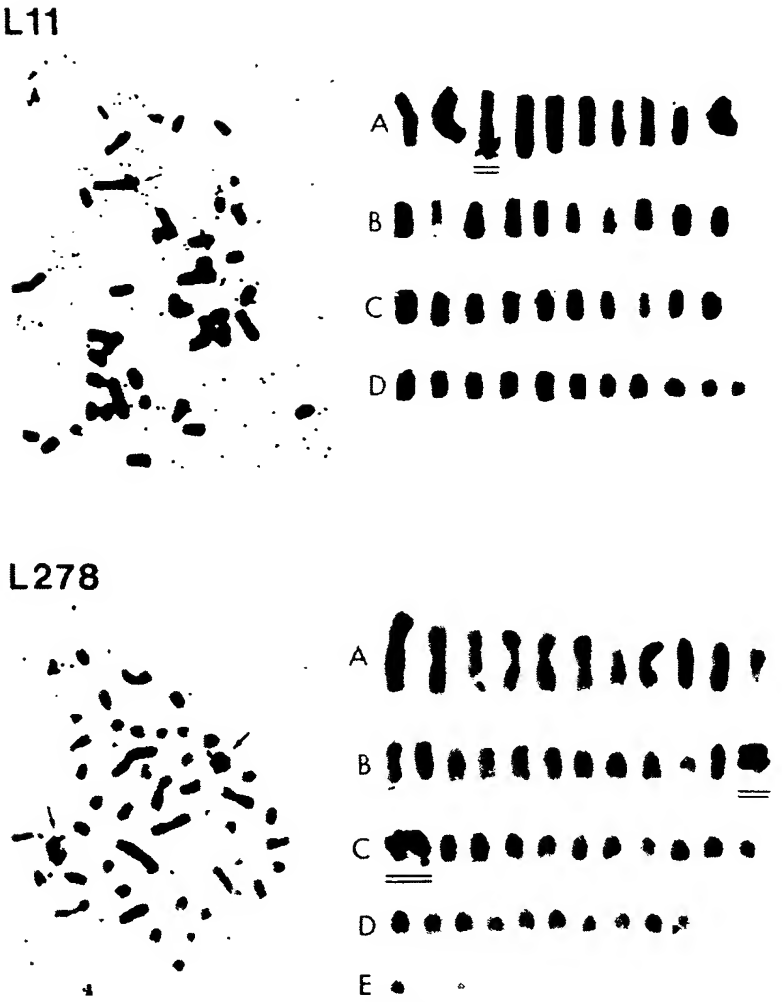

FIG. 5. In situ localization of the donated genes in transformants L11 and L278. Metaphase spreads of L11 and L278 were prepared as described in the text and hybridized with probes made by nick translation of recombinant HSV-tk gene-containing plasmids with either [^{125}I]dCTP (L11) or [^{3}H]dTTP (L278) to a specific activity of $\sim 5 \times 10^7$ cpm/μg. Each slide was hybridized overnight with 2.5×10^5 cpm of probe in a total volume of 25 μl in the presence of 10% sodium dextran sulfate 500. The slides were washed and exposed at 4°C for approximately 2 weeks. The left side of the figure shows the spreads from which the chromosomes on the right side were excised; these chromosomes are arranged according to size. Significant hybridization to the indicated chromosome of L11 (row A) was observed in six of six metaphase preparations examined. For L278, 20 of 25 metaphase preparations showed the chromosome in row B, and 15 of 25 showed the chromosome in row C. In spreads with fewer grains these chromosomes were clearly of different sizes.

mants from the predicted 1:6:1 ratio may have reflected stochastic fluctuations from randomness in the interspersion patterns of the A and B vectors within the concatemers. Such fluctuations were anticipated since these transformants did not contain large numbers of plasmid DNA copies. For example, a further analysis of the transformant shown in Fig. 7, lane a, indicated that this transformant contained three copies of the pBR322/ASV-2LTR/tk vectors in the arrangement A-A-B. Alternatively, the deviation in the ratio may have reflected more complex mechanisms for generating the head-to-tail concatemers, which would have included combinations of homologous recombination and amplification mechanisms.

We estimated the relative frequency with which homologous recombination occurred within the HSV-tk gene compared with the pBR322/ASV-2LTR sequences by performing the following analysis. Genomic DNA was cleaved with *Hin*dIII, which cut asymmetrically within the pBR322/2LTR sequences, or with *Bgl*II, which cut asymmetrically within the

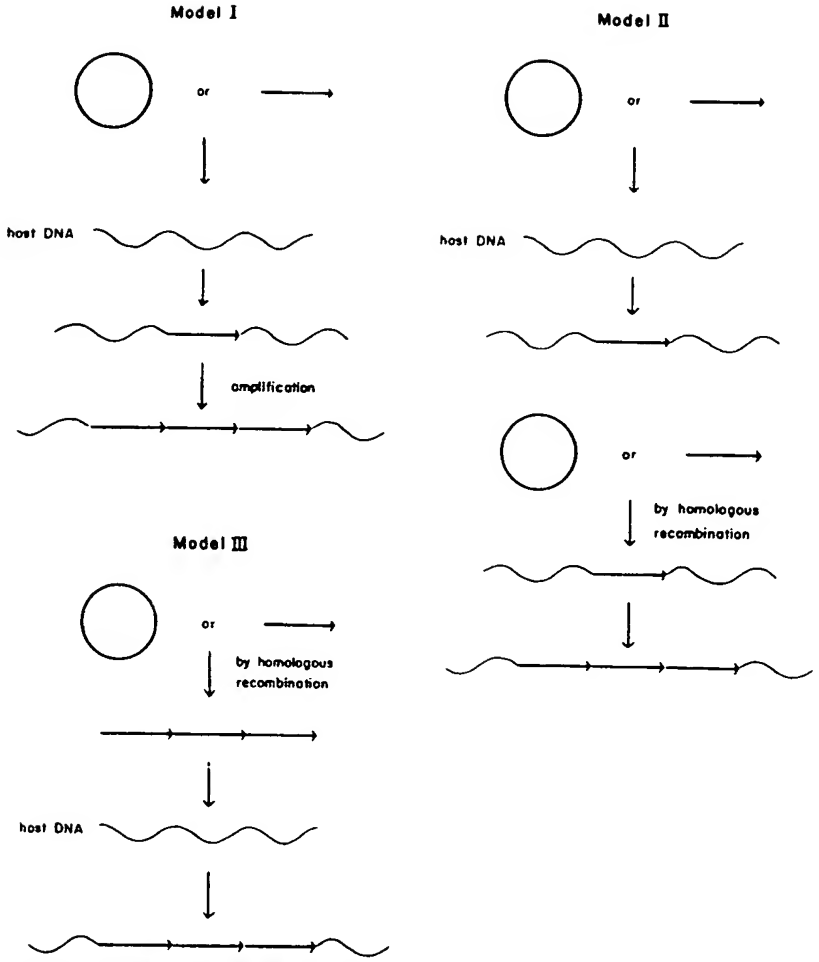

FIG. 6. Models for generating the head-to-tail concatemers. In model I a plasmid sequence is first integrated into host DNA and then amplified to generate a head-to-tail concatemer. In models II and III the head-to-tail concatemers are generated by a mechanism involving homologous recombination. In model II a single plasmid molecule is integrated into the host chromosome, and subsequent plasmid molecules then integrate at the same site by homologous recombination to generate the head-to-tail concatemer. In model III the head-to-tail concatemer is generated by homologous recombination before its integration as a unit into the host chromosome.

HSV-tk gene sequences. Such cleavages generated unique hybridization patterns depending on the site of recombination. If homologous recombination between A and B molecules occurred within the HSV-tk gene, then cutting with *Bgl*II resulted in a fragment of unit length, but cutting with a restriction enzyme that cut in the pBR322/ASV-2LTR sequence (*Hin*dIII) generated fragments of nonunit length (10.7 and 4.2 kb). Similarly, if homologous recombination occurred within the pBR322/ASV-2LTR sequence, cutting with *Hin*dIII resulted in a fragment of unit length, whereas cutting with *Bgl*II resulted in fragments of nonunit length (9.3 and 5.6 kb). Figure 7, lanes i through l show Southern transfers of *Hin*dIII digests of the same transformants shown in Fig. 7, lanes d through g (i.e., *Bgl*II digests). In addition to the major hybridizing band of unit length (7.4 kb) we also observed bands at 10.7 and 4.2 kb. Thus, recombination appeared to have occurred in both the HSV and the pBR322/ASV-2LTR sequences. Similar results were obtained when supercoiled or linear A and B vectors were coinjected. With transformant L351 (Fig. 7, lanes d and i) recombination occurred with equal frequency in the HSV and pBR322/ASV-2LTR sequences. With transformants L352, L353, and L354, we observed a bias toward more recombination in the pBR322/ASV-2LTR region of the plasmid. This bias may reflect stochastic fluctuations within the small sample size or preferential sites of recombination.

In two of the *Hin*dIII digests (Fig. 7, lanes k and l) we also observed a plasmid dimer band (14.9 kb). This band could have arisen either

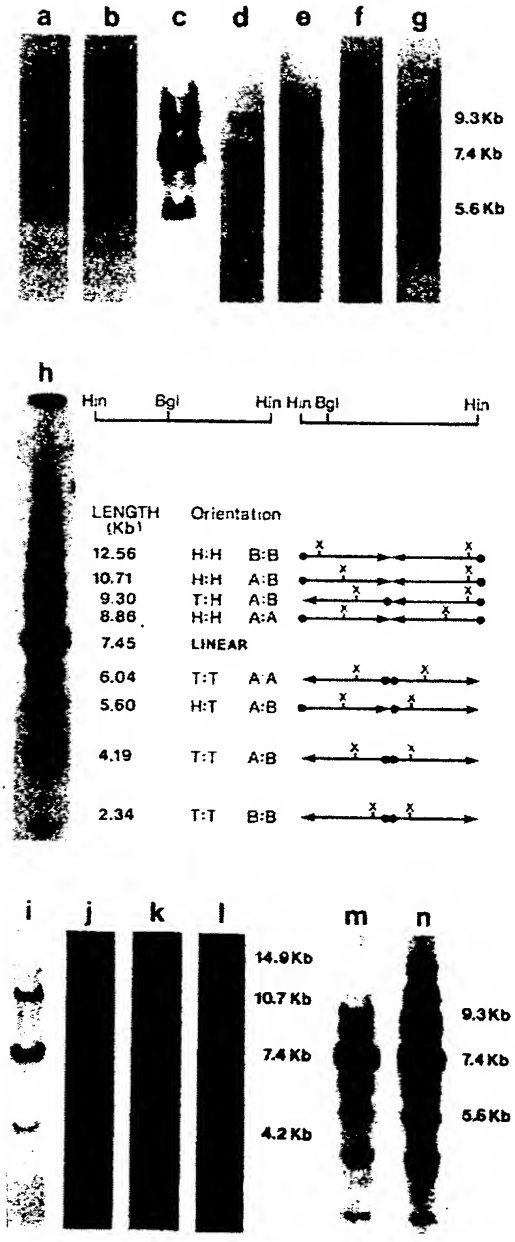

FIG. 7. Southern transfer analysis of transformants obtained by coinjecting pBR322/ASV-2LTR/tk A and B DNAs. Transformants L301, L302, and L303 (lanes a through c, respectively) were obtained by coinjecting 25 copies of both pBR322/ASV-2LTR/tk A and B supercoiled DNAs. Genomic DNAs from these transformants were hydrolyzed with *Bgl*II and processed by the method of Southern (33). In addition to the major hybridizing band of unit length (7.4 kb), we also observed bands which were 9.3 and 5.6 kb long. These latter bands arose from adjacent A and B vectors that recombined in the pBR322/ASV-2LTR sequences (see text). Transformants L351, L352, L353, and L354 (lanes d through g, respectively) were obtained by coinjecting approximately 15 copies of both pBR322/ASV-2LTR/tk A and B linear DNAs (linearized with *Hin*dIII). Genomic DNAs from these transformants were hydrolyzed with *Bgl*II. The hybridization patterns are very similar to those obtained by coinjecting A and B supercoiled DNAs. Lane h shows the Southern transfer pattern of a *Bgl*II digest of an in vitro synthesized concatemer in which the A and B vectors were randomly oriented (see text). A schematic diagram for the origin of each *Bgl*II fragment is provided. Lanes i through l show the Southern transfer patterns of *Hin*dIII digests of genomic DNA from transformants L351, L352, L353, and L354, respectively (see above). In addition to the major hybridizing band of unit length (7.4 kb), we also observed bands which were 10.7 and 4.2 kb long. These bands arose from adjacent A and B vectors that recombined in the HSV-tk gene containing *Bam*HI sequence (see text). Transformants L356 and L357 (lanes m and n, respectively) were obtained by coinjecting 75 copies of each pBR322/ASV-2LTR/tk A and B linear DNA. The Southern transfer pattern of the *Bgl*II digest of these transformants is similar to the marker pattern (lane h). A quantitative examination of these blots indicated that a majority of the A and B vectors within the concatemers were still oriented head to tail. Hin, *Hin*dIII; Bgl, *Bgl*II; H:H, head to head; H:T, head to tail; T:T, tail to tail.

from the loss of a *Hin*dIII site during incorporation of linear A and B molecules into the concatemer or from incomplete digestion of genomic DNA with *Hin*dIII. The ratio of monomer to dimer bands did not change after genomic DNA was digested with two- and fourfold excess concentrations of *Hin*dIII. The other two transformants (Fig. 7, lanes i and j), which were also obtained by coinjecting A and B vectors linearized with *Hin*dIII, did not exhibit plasmid dimer bands.

The results of the experiments discussed above argue strongly that the head-to-tail concatemers are generated by homologous recombination; however, these experiments did not distinguish between models II and III. Formal distinction between models II and III is indeed difficult because these two models utilize the same enzymatic machinery and differ only in the kinetic appearance of the postulated intermediates.

Stability of the head-to-tail concatemers. Since the head-to-tail concatemers appeared to be formed by homologous recombination, it was of interest to determine whether these sequences were stable after integration into the host chromosome. It was possible that these concatemers would be lost readily by a mechanism which used, for example, the same enzymatic machinery that led to their formation.

Three transformants containing head-to-tail tandem repeats were grown in nonselective medium for 50 generations. The stability of the concatemers was measured periodically by Southern transfer analysis, which estimated the

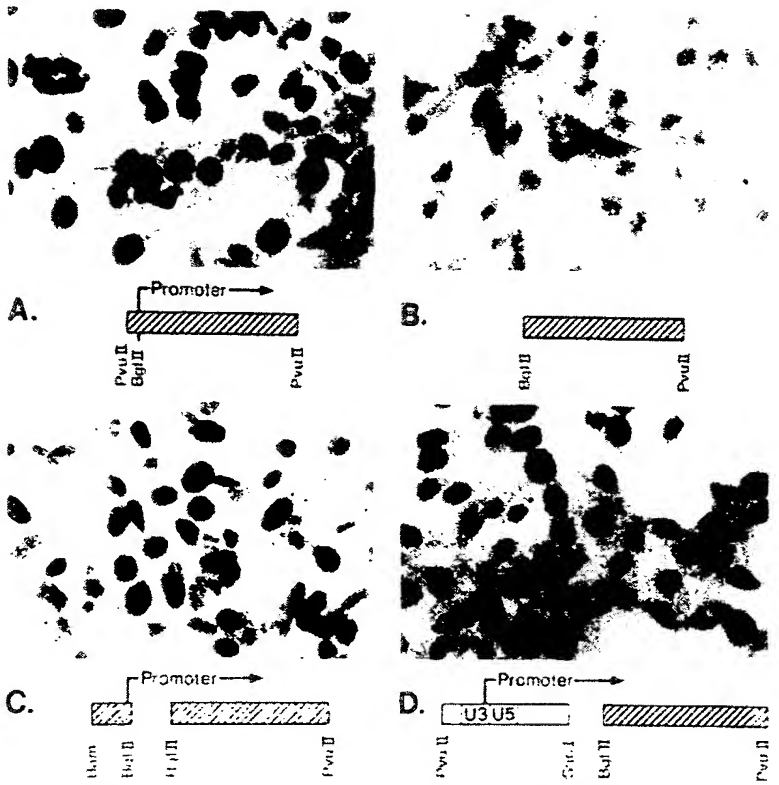

FIG. 8. Autoradiographic analysis for thymidine kinase activity after injection of the designated HSV-tk gene-containing and ASV LTR DNA fragments. After injection of the designated DNA fragments, the cells were incubated in minimal essential medium containing 10% fetal calf serum for 24 h at 37°C in a 5% CO_2 incubator to allow for the expression of HSV-tk genes. The thymidine kinase activity present in each cell was determined by measuring the capacity of the cells to incorporate [^{3}H]thymidine into DNA. The cells were incubated for 16 h at 37°C in minimal essential medium containing 10% fetal calf serum and 25 μCi of [^{3}H]thymidine per ml. After incubation in [^{3}H]thymidine-containing medium, the cells were washed, fixed, and processed for autoradiography as previously described (9). A total of 25 to 30 cells in each of the fields received an injection of the DNA fragments. Bam, *Bam*HI.

average number of plasmid DNA copies per cell, and by in vivo [^{3}H]thymidine incorporation into DNA, followed by autoradiography, which measured the fraction of cells which retained HSV-tk gene enzymatic activity. Over the duration of the experiment there was no detectable loss of plasmid sequences, and no increase in the fraction of TK$^-$ cells was observed.

End-to-end ligation. We observed that if large quantities of linear A and B vectors (i.e., more than 75 copies of each per cell) were coinjected, then some random end-to-end ligation was detected (Fig. 7, lanes m and n). The Southern transfer pattern for these transformants was very similar to the marker pattern (Fig. 7, lane h) generated by ligation of the two vectors in vitro. A careful examination of these Southern transfers indicated that the transformants contained concatemers composed of A and B vectors which were predominantly in the head-to-tail orientation mixed in with randomly oriented A and B vectors.

We examined the substrate requirements for this end-to-end ligation in vivo by coinjecting two fragments (one bearing the coding sequence for the HSV-tk gene and the other bearing a promoter sequence) and measuring HSV-tk gene enzymatic activity (Fig. 8). Approximately 50 promoter fragments were coinjected with 25 HSV-tk gene-containing *Bgl*II-*Pvu*II fragments. We made the assumption that in order to make a functional transcript, the two fragments had to be correctly ligated in the recipient cell. Quantitation for this experiment is shown in Fig. 9, along with diagrams of the coinjected fragments. The results are presented as the ratio of the number of cells exhibiting HSV-tk gene activity to the number of cells receiving injections with the respective fragment(s). As a positive control, cells were injected with the HSV-tk gene-

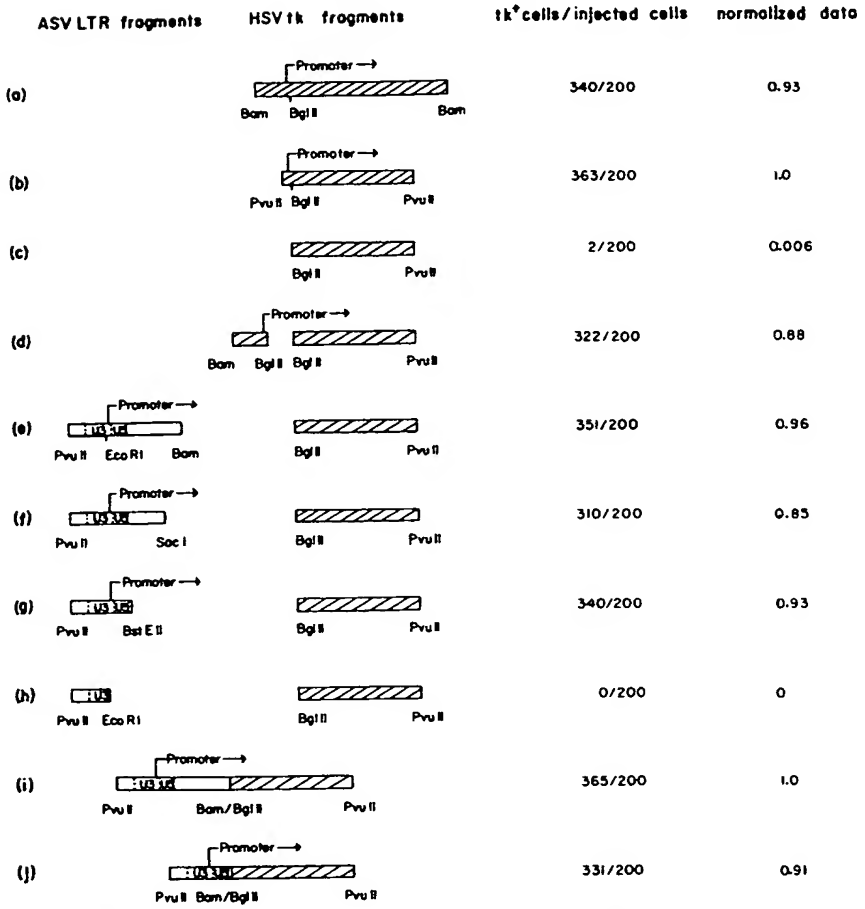

FIG. 9. Number of TK^+ cells after injection of 3T3tk$^-$ cells with HSV-tk gene-containing and ASV LTR DNA fragments. The thymidine kinase activity present in each cell was determined autoradiographically as described in the legend to Fig. 8. The number of TK^+ cells could exceed the number of injected cells because the [^{3}H]thymidine incorporation assays were done 24 h after injection; by this time many of the cells had divided. Bam, *Bam*HI.

containing *Bam*HI and *Pvu*II fragments, both of which contained the intact HSV-tk gene promoter and the HSV-tk structural gene. The number of TK^+ cells exceeded the number of cells receiving an injection (Fig. 9a and b) because the [^{3}H]thymidine incorporation assays were done 24 h after injection, by which time many of the cells had divided. Very few TK^+ cells were observed among the cells that received injections of the control HSV-tk gene-containing *Bgl*II-to-*Pvu*II fragment (Fig. 8B and 9c), which lacked a functional promoter (25). Coinjection of the HSV-tk gene-containing *Bam*HI-to-*Bgl*II fragment, which contained the HSV-tk gene promoter, with the HSV-tk gene-containing *Bgl*II-to-*Pvu*II fragment resulted in many TK^+ cells, indicating that a functional ligation of the two fragments occurred (Fig. 8C and 9d).

When we began these experiments, we knew that the ASV LTR could be fused to the HSV-tk gene at the *Bgl*II site and function as an effective promoter (P. Luciw and M. R. Capecchi, unpublished data). In transformants obtained by injecting the fused gene, Luciw detected a hybrid ASV-5′–HSV-tk gene message which was initiated at or very near the ASV cap site (data not shown). The point of fusion could be at the ASV *Bam*HI or *Bst*EII site (Fig. 1 and Figs. 9i and j), indicating that the length of the leader sequence did not affect the activity of the resulting transcript.

With these results in mind, we could test whether the in vivo end-to-end ligation reaction required homologous ends. The ASV LTR fragment contains *Bam*HI, *Sac*I, and *Bst*EII sites in the leader. The *Sac*I and *Bst*EII restriction sites show no homology to the *Bgl*II site. Furthermore, *Sac*I generates a 3′ overhang, whereas *Bgl*II generates a 5′ overhang. As shown in Fig. 9e through g, ASV LTR fragments which termi-

nated at the *Bst*EII, *Sac*I, and *Bam*HI sites were all ligated efficiently to the *Bgl*II-to-*Pvu*II HSV-tk gene-containing fragment to form a functional gene. As an additional control, the ASV LTR fragment terminating at the *Eco*RI site was coinjected with the HSV-tk gene-containing *Bgl*II-to-*Pvu*II fragment. The *Eco*RI site in the ASV LTR sequence is upstream (5') of the TATA box (35). No TK^+ cells were observed in cell preparations that received injections of these two fragments (Fig. 9h). Also, no TK^+ cells were observed among cells that received injections of only promoter fragments (data not shown).

From the experiments described above, it is clear that two DNA fragments can be ligated together in mammalian nuclei irrespective of the sequence or topology at their ends. Since any two DNA fragments appear to be joined by ligation, many of the ligation reactions that occurred in the above-described experiments must have been nonfunctional (e.g., promoter fragments that were joined together, HSV-tk gene-containing *Bgl*II-*Pvu*II fragments that were joined together, promoter fragments that were joined downstream to the HSV-tk gene-containing *Bgl*II-*Pvu*II fragment, etc.). In fact, if any two fragments can be joined together, then only 1 in 12 possible ligation reactions would be expected to be productive. Since we observed a productive ligation in nearly every cell that received an injection, the efficiency of joining two free ends of any DNA molecules together is extremely high.

DISCUSSION

The ability of cultured mammalian cells to incorporate exogenous DNA into their chromosomes can be remarkably efficient when DNA is injected directly into the nucleus through glass micropipettes. We have obtained transformation efficiencies greater than 20% by injecting as few as three linear molecules per cell. As discussed below, the transformation efficiency reflects the physical form of the injected molecules, the presence of enhancer sequences, and the type of recipient cells used. The number of gene copies integrated into the genome can be varied by simply altering the quantity of DNA injected. Using this method, we have obtained transformants containing a single copy or as many as 50 to 100 copies of the donated genes. Although the number of gene copies which are stably integrated is roughly proportional to the number of gene copies injected, there is considerable variation from transformant to transformant. This fluctuation may indicate that there are periods of the cell cycle during which exogenous DNA can be incorporated into the genome more efficiently.

As demonstrated by in situ hybridization, multiple copies of the transforming DNA are integrated at one or a very few sites in the host chromosome, and the site of integration is different in independent transformants. Robins et al. (30) and de Saint Vincent et al. (15) previously reached the same conclusion for transformants obtained by calcium phosphate coprecipitation and protoplast fusion, respectively. These results suggest that a rate-limiting step in the DNA-mediated transformation process may be the insertion of exogenous DNA into a host chromosome. It will be of interest to determine whether the lower transformation efficiency of RAT-2tk$^-$ cells can be correlated to this rate-limiting step.

In more than 100 transformants we have never found free plasmid DNA as supercoiled, linear, or relaxed circular molecules. Such molecules would have been detected by Southern transfer analysis of either undigested genomic DNA or genomic DNA digested with a restriction enzyme that does not cut the plasmid DNA.

We have investigated the process by which the donated molecules become integrated into the host chromosome by varying the numbers and structures of the injected molecules. When the cell nucleus is presented with supercoiled molecules, no preferred site of integration with respect to plasmid sequences is observed in transformants containing a single copy of the plasmid. Single linear molecules, on the other hand, appear to be inserted into the host genome through their ends. In the process of insertion, the restriction sites at the ends of the linear molecule are lost, but a site as few as six nucleotides from the terminus is found intact.

A number of other observations also indicate that the DNA injected into nuclei of LMtk$^-$ or RAT-2tk$^-$ cells is not extensively hydrolyzed or rearranged before or during integration into the host chromosome. Within the resolution of the Southern transfer technique, analyses of a number of single-copy transformants have indicated that the sum of the lengths of internal fragments is equal to the length of the intact plasmid. Furthermore, DNA from a number of transformants containing multiple copies of the plasmid vector have been analyzed by digestion with a series of restriction enzymes. Aside from the expected junction fragments, we have not frequently observed spurious hybridizing bands that cannot be accounted for by restriction maps of the vectors. Also, in transformants obtained by injecting multiple copies of a plasmid DNA linearized with a particular restriction enzyme, the loss of the restriction site of the enzyme in the concatemer is less than 10%.

When more than 5 linear or 25 supercoiled molecules are injected per cell, the transformants contain multiple copies of the donated sequences in a head-to-tail array. By analyzing

transformants obtained by coinjecting two vectors which are identical except that in one a portion of the vector sequence is inverted, we have been able to conclude that the head-to-tail concatemers are generated predominantly by homologous recombination and that recombination can occur throughout the vector.

The interspersion of the A and B vectors in the same concatemer demonstrates that homologous recombination can account for the formation of the head-to-tail concatemers but does not rule out the possibility that amplification is a contributing mechanism. However, the additional observation that the number of gene copies integrated into the host chromosome is roughly proportional to the number of gene copies injected into the recipient cells argues against frequent amplification of the donated DNA sequences. Furthermore, we have not observed transformants containing many copies of the donated plasmid sequences after injection of only a few copies of the plasmid DNA.

Head-to-tail concatemers of papovavirus DNA have been observed after transfection of nonpermissive cells with SV40 or polyoma virus DNA (4, 7, 12, 13, 26). Replicative synthesis of the viral DNA has been implicated as the mechanism for generating these concatemers. Our results differ from the results of other workers (4, 7, 12, 13, 26) in that the plasmid sequences which we injected were presumed to be replicatively incompetent.

It is interesting that when small numbers of plasmid molecules are injected into the recipient, linear molecules transform LMtk$^-$ and RAT-2tk$^-$ cells much more efficiently than supercoiled molecules. This finding implies that a DNA molecule with exposed ends is a better substrate for the recombination machinery of the cells. Consistent with this hypothesis are the studies with single-copy transformants which suggest that linear molecules are incorporated into the host chromosome through their ends. We have also observed that linear molecules can be recruited into concatemers by homologous recombination. The simplest interpretation of this observation is that many of the linear molecules must be recircularized before entry into the concatemer. Finally, if the concentration of linear molecules in the nucleus is high (~100 copies per cell), one even observes end-to-end ligation.

Therefore, it appears that in the nucleus competition exists for the ends of the donated DNA to be ligated into host DNA sequences, to themselves (resulting in recircularization), or to other exogenous DNA sequences (resulting in end-to-end ligation). Which event occurs is presumably controlled by the concentration of ends and the enzymatic machinery which mediates each reaction. The observation that linear molecules are a better substrate for transformation can be explained if linear molecules are an obligatory intermediate for incorporating exogenous DNA into the host chromosome. Consistent with this hypothesis is the observation that a high transformation efficiency can be obtained by using fewer than five supercoiled molecules when these molecules are coinjected with a few copies of any linear molecule containing homology to the supercoiled molecule (Capecchi, unpublished data). It will be of interest to determine whether the linear molecules in these transformants are found at the ends of the concatenate. A similar prediction of such a model is that the junction between the host chromosome and the head-to-tail concatemers generated by injecting linear molecules is at the ends of the linearized plasmid.

After injection of approximately 100 plasmid molecules per cell, we have isolated transformants in which as many as 50 to 100 plasmid molecules have been incorporated into the head-to-tail concatemer. This propensity for generating head-to-tail concatemers indicates that the cells have a very active enzymatic system for homologous recombination. It will be interesting to determine whether we can exploit this machinery to "target" a gene by homologous recombination to a specific chromosomal location. On the other hand, the very efficient joining of two DNA fragments in vivo irrespective of their sequence compositions may reflect the presence in somatic cells of a very active illegitimate or nonhomologous recombination system. The success of gene targeting experiments will clearly depend on the interplay of these two recombination systems. Pertinent to such experiments are the observations of Graessmann et al. (16) and Botchan et al. (8) that retransformation of revertant cells that still contain SV40 sequences leads to integration of SV40 at separate chromosomal sites and that integration of SV40 appears to involve homologous recombination, but only between a very limited number of nucleotide base pairs (34).

One model for generating head-to-tail concatemers with linear molecules which we have not addressed is the following: initially plasmid sequences could be recruited into a concatemer randomly by end-to-end ligation. However, due to the possible instability of long inverted repeats within the host genome, adjacent head-to-head or tail-to-tail plasmid sequences could be selectively removed, leading to the final head-to-tail concatemers. Such models are rendered unnecessary by our finding that concatemers which contain randomly oriented plasmid sequences (e.g., L356 and L357) are stable.

In theory, the homologous recombination ma-

chinery which forms head-to-tail concatemers could also be used to excise these concatemers. Thus, it was of interest to determine whether such structures are as unstable as their counterparts in bacteria (1). We have shown that for a number of transformants the sequences within the head-to-tail concatemers are stable even when the cells are grown under nonselective conditions. Varmus et al. (39) have reported excision of Moloney murine leukemia virus DNA from cellular DNA apparently by a homologous recombination event between the viral LTR sequences. The estimated frequency of this event was 10^{-6} to 10^{-7} per generation. Our method for estimating the instability of the head-to-tail concatemers would not have detected such a rare event. Head-to-tail duplications of SV40 and polyoma virus sequences in cultured mammalian cells appear to be less stable (11, 20, 22). A simple explanation of this difference would be that these sequences are excised by a mechanism which is dependent upon both host and viral factors (e.g., T-antigen in the excision of polyoma virus duplications [4]). On the other hand, eucaryotic organisms have probably evolved a mechanism for maintaining repeated sequences, such as ribosomal genes, even though such genes are clearly capable of undergoing amplification (37) and rearrangement (21). The stability of our head-to-tail concatemers may reflect such a mechanism.

In addition to the structure of the injected molecules, another parameter that strongly affects the transformation frequency is the presence or absence of enhancer sequences. The SV40 DNA sequence which enhances the DNA-mediated transformation frequency has been mapped to the 72-base pair (bp) repeat sequence located between nucleotides 107 and 250 of the SV40 late region (Luciw and Capecchi, unpublished data). In Fig. 1 we show that the SV40 *Hin*dIII-to-*Hpa*II fragment, which contains the SV40 origin of DNA replication, the 72-bp repeat, and the 21-bp repeat (located between the SV40 origin of DNA replication and the 72-bp repeat) contains the transformation enhancer activity. On the other hand, *Eco*RII fragment G, which contains the SV40 origin of DNA replication, the 21-bp repeat, and only part of one of the 72-bp repeat, shows no detectable enhancing activity. The SV40 *Nco*I fragment, which contains the 72-bp repeat and the 21-bp repeat but not the SV40 origin of DNA replication, exhibits full enhancer activity. Taken together, these results localize the transformation enhancer activity to a region surrounding the 72-bp repeat.

The same SV40 72-bp repeat has been shown to be required for transcription of SV40 T-antigen (6, 19) and to enhance transcription from a rabbit β-globin gene 100-fold (5). As in the DNA-mediated transformation assay, the amount of enhanced transcription is not critically dependent on the position of the 72-bp repeat within the rabbit β-globin recombinant plasmid. These observations invite speculation on the relationship between the enhancing effects of the same sequences on two different phenomena, DNA-mediated transformation and transient expression of certain genes in transfected cells.

Enhancer sequences may directly or indirectly stimulate recombination (for example, by making the recombinant plasmid a better substrate for recombination or by shuttling the recombinant plasmid to a cellular compartment with a high recombination activity). Alternatively, enhancer sequences may stimulate DNA-mediated transformation not by increasing the frequency of integration into the host chromosome, but by increasing the probability that a gene is active after its integration into the host chromosome. A gene with an associated enhancer sequence may be transcriptionally active in a normally silent chromosomal environment. In the latter model enhancer sequences may function by altering local chromatin structure so as to facilitate transcription of an associated gene. It is possible to distinguish between models in which the enhancer sequences affect integration and models that affect expression of the transforming gene after integration by measuring the incorporation of the exogenous sequences into the host chromosome in the absence of selection.

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